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D B Peck

Name (Print)

Signature

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: David Berd

Serial No.: 09/304,859 Art Unit: 1642

Confirmation No.:

Filed: May 4, 1999 Examiner: J. Hunt

For: COMPOSITION COMPRISING TUMOR CELL AND EXTRACTS AND METHOD OF USING THEREOF

DECLARATION OF DAVID BERD, M.D.
UNDER 37 C.F.R. § 1.132

Hon. Commissioner of
Patents and Trademarks
Washington, DC 20231

Sir:

I, David Berd, M.D., hereby declare and state that:

1. I am the inventor of the invention claimed in the above-captioned patent application.

2. I presently hold the position of Professor of Medicine, Director of Oncology Laboratory, Jefferson Medical College of Thomas Jefferson University, Philadelphia, Pennsylvania,

where I have been employed since 1984. A copy of my Curriculum Vitae is attached as Exhibit 1.

3. I reaffirm my duty of candor and good faith in dealing with the Office, including the duty to disclose to the Office all information known to be material to the patentability of the invention.

Unexpected Features and Advantages of this Invention

I have reviewed the outstanding Office Action in connection with this patent application. I understand that the Examiner has rejected the claims on the basis of a combination of U.S. Patent No. 5,290,551 with a second reference by Elliot et al. (Patent No. 5,478,556). The Elliot patent describes an immunization schedule designed to elicit an antibody response, which is unrelated to immunotherapy of cancer. My U.S. Patent (and scientific publications prior to the Abstracts and article attached to this Declaration as Exhibit 2, 3, 4, and 5) reports a 28 day cycle for administration of the haptenized tumor cell vaccine. The reason we selected the 28 day cycle for immunization was to avoid administering cyclophosphomide (CY) on a more frequent basis than monthly as the Examples and published protocols called for cyclophosphomide administration immediately prior to each vaccination. Our clinical protocols were subsequently modified to include cyclophosphomide administration prior to the first vaccination, but not subsequently. Only upon this modification could we administer haptenized tumor cell vaccines on a weekly basis. Indeed, the unexpected discovery that one could omit CY treatment prior to every vaccine and achieve superior results with weekly vaccine administration underlies this invention. We could not predict that weekly administration would be as effective as the 28 day period administration, much less that it would be more effective in certain circumstances (compare Group A to Group B in the Abstract attached as Exhibit 2).

5. Additional research (the results of which are the subject of a pending patent application) has revealed that an induction dose of haptenized tumor cells enhances effectiveness of the immunotherapy. These data are reported in the Abstract attached as Exhibit 5. These results explain why the group who received weekly administration of the vaccine over a twelve week period did not show as good results as the group that received it over the six week period (see Exhibit 2); the former group did not receive what has subsequently been discovered to be an induction dose at the appropriate time prior to administration of cyclophosphomide. These data confirm that a weekly vaccine administration regimen provides as good or even better anti-tumor reponses, which is clearly an advantage to the patients.

6. While the references cited by the Examiner fail to establish any predictability of weekly administration, the data provided in the Abstracts attached at Exhibits 2 and 5 clearly demonstrate that any changes in dosage regimen and timing has unpredictable effects on vaccine efficacy. Indeed, the variable results in the entire history of tumor immunotherapy support this. Accordingly, the discovery that weekly administration of a haptenized tumor vaccine was as or more effective than the 28 day cycle of administration was unpredictable.

Unavailability of Certain References as "Prior Art"

7. I am a co-author, along with M.J. Mastrangelo, E. Bloome, W. Medley, C. Clarke and H.C. Maguire of an Abstract from the Proceedings of the Thirty-Third Annual Meeting of the American Society of Clinical Oncology, Volume 7, page 438a, Abstract No. 1570 (May 7-20, 1997) entitled "Delayed-type hype sensitivity (DTH) responses induced by autologous, hapten-modified melanoma vaccine— importance of dosage schedule," a copy of which is attached as Exhibit 3.

I am the only inventor of the subject matter claimed in the above-captioned patent application and disclosed in the referenced Abstract. Based on personal knowledge and information and belief, the contributions of each of the above-named co-authors is as follows:

a. Michael J. Mastrangelo, M.D., Professor of Medicine, Thomas Jefferson University provided patient samples and assisted in the clinical care of patients

b. Ellen Bloome, R.N. administered vaccines and monitored patients under my direction and control.

c. Winifred Medley prepared the vaccines and skin testing materials under my direction and control.

d. Carmella Clarke prepared the vaccines and skin testing materials under my direction and control.

e. Henry C. Maguire, M.D., Professor of Medicine, Thomas Jefferson University, as been a collaborator and advisor. On this project, he provided advice in connection with cyclophosphomide treatment in conjunction with tumor vaccination, and with respect to the delayed-type hypersensitivity (DTH) response. Dr. Maguire has expertise in both areas and we have collaborated previously on studies concerning cyclophosphomide treatment and DTH (*see e.g.* Berd et al., 1986, "Induction of Cell-mediated Immunity to Autologous Melanoma Cells and Regression of Metastases after Treatment with a Melanoma Cell Vaccine Preceded by Cyclophosphomide," Cancer Research 46:2572-2577, a copy of which is attached

as Exhibit 6). Dr. Maguire also assisted in the skin testing and DTH measurements described in the Abstract.

My co-authors, therefore, only assisted in routine experimentation and in experimentation unrelated to the invention disclosed and claimed in the above-captioned patent application. These contributions were made at my request and direction, and under my control. No contribution to the idea or conception of the subject matter of any claim was provided by these co-authors.

8. I am also co-author, along with Henry C. Maguire, Jr., Lynn M. Schechter, Ralph Hamilton,Walter W. Houck, Takami Sato, and Michael J. Mastrangelo of an article published in the Journal of Clinical Oncology, Vol. 15, No. 6 (June) 1997, pp. 2359-2370 entitled "Autologous Hapten-Modified Melanoma Vaccine as Postsurgical Adjuvant Treatment After Resection of Nodal Metastases," a copy of which is attached as Exhibit 4. I am the only inventor of the subject matter claimed in the above-captioned patent application and disclosed in this article. Based on personal knowledge and information and belief, the contributions of each of the above-named co-authors is as follows:

a. Henry C. Maguire contributed as in ¶ 7. d., above.

b. Lynn M. Schechter referred patients to the study.

c. Ralph Hamilton referred patients to the study.

d. Walter D. Houck assisted with statistical analysis of the data from the study.

e. Takami Sato referred patients to the study.

f. Michael J. Mastrangelo contributed as described in ¶ 7. a., above.

My co-authors, therefore, only assisted in routine experimentation and in experimentation unrelated to the invention disclosed and claimed in the above-captioned patent application. These contributions were made at my request and direction, and under my control. No contribution to the idea or conception of the subject matter of any claim was provided by these co-authors.

9. I am also co-author, along with J. Carlson, E. Bloome, W. Medley, and C. Dunton of an Abstract from the Proceedings of the American Association for Cancer Research, Volume 39, page 356, Abstract No. 2431 (March 1998) entitled "Introduction of a delayed-type hypersensitivity (DTH) to ovarian cancer cells after treatment with autologous (AUT), hapten-modified vaccine," a copy of which is attached as Exhibit 5. I am the only inventor of the subject matter claimed in the above-captioned patent application and disclosed in the referenced Abstract. Based on personal knowledge and information and belief, the contributions of each of the above-named authors is as follows:

a. John Carlson referred patients to the study.

b. Ellen Bloome, R.N. administered vaccine and monitored patients under my direction and control.

c. Winifred Medley prepared the vaccines and skin testing materials under my direction and control.

d. Charles Dunton referred patients to the study and assisted in writing the

clinical protocol, but not in developing the weekly administration strategy.

My co-authors, therefore, only assisted in routine experimentation and in experimentation unrelated to the invention disclosed and claimed in the above-captioned patent application. These contributions were made at my request and direction, and under my control. No contribution to the idea or conception of the subject matter of any claim was provided by these co-authors.

10. I further declare that all statements made in this Declaration of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful, false statements and the like so made are punishable by fine or imprisonment, or both, under Section 100 1 of Title 18 of the United States Code, in that such willful, false statements may jeopardize the validity of the application or any patent issuing thereon.

DAVID BERD, M.D.

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June / /, 2001

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May, 2001

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Date of Birth:

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Marital Status:

Married 1968
Children: Brendan Daniel, 1976
Deborah Laurie, 1978

Education:

1963-66 B.S., 5 year cooperative medical program, Pennsylvania State University

1964-68 M.D., Jefferson Medical College

Postgraduate Training and Fellowship Appointments:

1968-69 Internship, straight medical, Hospital of the University of Pennsylvania, Philadelphia, Pa.

1969-70 Residency, internal medicine, Hospital of the University of Pennsylvania, Philadelphia, Pa.

1972-74 Fellowship, medical oncology, Yale University School of Medicine, New Haven, CT

1974-75 Research Fellowship, funded by NIH Special Fellowship, Yale University School of Medicine

Military Service:

1970-72 U.S. Public Health Service, Center for Disease Control, Laboratory Branch, Mycology Section, Atlanta, Ga.

Certification:

1974 American Board of Internal Medicine

1975 Medical Oncology

Licensure:

1969 Pennsylvania #011257

Academic Positions:

1991-present	Member, Jefferson Cancer Institute
1991-present	Professor of Medicine, Jefferson Medical College of Thomas Jefferson University
1984-91	Associate Professor of Medicine, Jefferson Medical College of Thomas Jefferson University
1984 - 1991	Director, Flow Cytometry Facility, Thomas Jefferson University
1975-84	Research Physician, Fox Chase Cancer Center
1978-84	Adjunct Assistant Professor of Medicine, University of Pennsylvania School of Medicine
1975-78	Clinical Associate in Medicine, University of Pennsylvania School of Medicine
1971-72	Instructor in Medicine, Emory University School of Medicine

Hospital Appointments:

1984 Attending Physician, Thomas Jefferson University Hospital

Honorary Societies:

1964 Phi Beta Kappa

1968 Alpha Omega Alpha

Honors:

January 15, 1997 - featured on cover of *Cancer Research*

May, 1997 - Outstanding Researcher Award, Department of Medicine, Thomas Jefferson University

Professional Societies:

1976 New York Academy of Sciences

1977 American Association for Cancer Research
Tellers Committee, 1992-93
Chairman, Membership Committee, 1993-94

1977 American Society for Clinical Oncology

1982 American Association for Advancement of Science

1984 Society for Biological Therapy

1985 Sigma XI

1985 Society for Analytical Cytology

1986 American Association of Immunologists

Government Advisory Groups:

1985-89 Member, Experimental Therapeutics Study Section (ET-1), NIH

1985 Ad Hoc Reviewer, Experimental Therapeutics Study Section, NIH

1987 Ad Hoc Contract Technical Review Group, Contracts Review Group, NCI

1989 Ad Hoc Review Committee, National Cooperative Anti-Cancer Model Development Groups

1990 Ad Hoc Review Committee, NIDDKD

1989 National Institutes of Health Reviewers Reserve (NRR)

1990 Ad Hoc Review Committee, National Institute of Diabetes and Digestive and Kidney Diseases

1990 Ad Hoc Review Committee, Prevention Clinical Trials Utilizing Intermediate Endpoints and their Modulation by Chemopreventive Agents

1991 Ad hoc member, Cancer Center Support Review Committee

1992 Ad Hoc Review Committee, Phase II Clinical Trials of New Chemopreventive Agents

1992 Experimental Therapeutics-2 Study Section, Ad Hoc Review

1994 DOD Breast Cancer Program, Clin-1 Study Section

1994 Ad Hoc Contracts Technical Review Group, Phase I Contracts

1995 Ad hoc member, Cancer Center Support Review Committee

1996 Special Review Committee, Therapeutic Clinical Trials of Malignancies

Grant Support:

Principal Investigator - NIH CA 39248 "Autologous Hapten-Modified Vaccine for Human Cancer," 5/1/88-present; current award 4/1/99-3/31/02

Sponsored Research Agreement, AVAX Technologies, 4/96-present

Clinical Research Agreement, AVAX Technologies, "A Prospective , Randomized, Open-Label, Comparative Clinical Trial in Post—Surgical Melanoma Patients with Either DNP-Modified Autologous Tumor Vaccine or Interferon Alpha-2b," 9/98-9/01

NIH/NCI - "Autologous Hapten-Modified Vaccine for Renal Cell Cancer," submitted 4/00

Patents

5,290,551 - Treatment of melanoma with a vaccine comprising irradiated autologous melanoma tumor cells conjugated to a hapten (March 1, 1994)

6,093,700 – A method of inducing an immune response using Vaccinia virus recombinants encoding GM-CSF

Editorial Boards

June, 2000 – Associate Editor, *Cancer Research*
1997-present – Associate Editor, *Cancer Immunology Immunotherapy*

PUBLICATIONS

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Maguire HC, BERD D, Lattime EL, McCue PA, Kim S, and Mastrangelo MJ: Phase I study of R24 in patients with metastatic melanoma including evaluation of imunologic parameters. Cancer Biotherapy Radiopharm., 13:13-23, 1998.

McClay,E.F., BERD D, Mastrangelo, M.J.: The Dartmouth regimen: Gone or going strong? Cancer Invest., 16:421-423, 1998.

Wachsberger, PR, Gressen EL, Bhala A, Bobyock SB, Storck C, Coss RA, BERD D, Hauck W, and Leeper DB: Variability in glucose transporter 1 levels in human melanoma. 1999, *submitted.*

Nathan FE, BERD D, Sato T, Mastrangelo MJ: Paclitaxel and tamoxifen: An active regimen for metastatic melanoma. Cancer, 88:78-87, 2000.

Aoyama T, Mastrangelo MJ, BERD D, Nathan FE, Shields CL, Shields JA, Rosato EL, Rosato FE, Sato T: Protracted survival after resection of metastatic uveal melanoma. Cancer, 89:1561-1568, 2000.

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BERD D, Sato T, Cohn H, Maguire HC Jr., Mastrangelo, MJ: Treatment of Metastatic Melanoma with Autologous, Hapten-Modified Melanoma Vaccine: Regression of Pulmonary Metastases. *Submitted*

Erdile LF, Smith D, BERD D: Whole cell ELISA for detection of tumor antigen expression in tumor samples. *Submitted.*

Wachsberger PR, Gressen EL, Bhala A, Bobyock SB, Storck C, Coss RA, BERD D, and Leeper DB: Variability in glucose transporter 1 levels and hexokinase activity in human melanoma. 2000, submitted.

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Reviews and Editorials

Mastrangelo MJ, BERD D, and Bellet RE: Critical review of previously reported clinical trials of cancer immunotherapy with non-specific immunostimulants. Ann NY Acad Sci 227:94-122, 1976

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Medical Grand Rounds, Thomas Jefferson University, April 30, 1987. Active Immunotherapy of Melanoma.

Fifth Annual Medical Laboratory Immunology Symposium, Williamsburg, Va., May 3-6, 1987: Augmentation of Human Immunity by the Cytotoxic Drug, Cyclophosphamide.

Philadelphia Cancer Research Association, June 23, 1987, Thomas Jefferson University, Active Immunotherapy of Malignant Melanoma.

Franklin Institute Science Museum, July 10, 1987, Tumor Immunology.

Second Conference on Immunity to Cancer. Williamsburg, November, 1987. Low doses of chemotherapy to inhibit suppressor T cells.

Medical Grand Rounds, Thomas Jefferson University, March 10, 1988, Post-Surgical Chemotherapy of Cancer - The Why's and the Why-Nots

Radiation Biology Seminar, Thomas Jefferson University, "Potentiation of Antitumor Response by Cyclophosphamide," March 18, 1988.

Immunotherapy of melanoma with autologous tumor vaccine preceded by low dose cyclophosphamide. UCLA Symposium on Human Tumor Antigens and Specific Tumor Therapy, 1988.

Co-Chairman, Minisymposium on Immunopotentiation by Chemotherapeutic Agents, Amer. Assoc. Cancer Res. meeting, New Orleans, LA, May, 1988.

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Radiation Biology Seminar, Thomas Jefferson University, "Induction of Cellular Immunity to Autologous Human Melanoma Cells", March 16, 1990.

National Cancer Institute Cancer Vaccine Workshop, participant, October 29-30, 1990, Bethesda, MD

Philadelphia Immunology Conference, Workshop Leader, October 31, 1990.

Cancer Center Conference, Medical College of Pennsylvania, December 14, 1990.

Tumor Conference, St. Claire's Hospital, Denville, NJ, January 14, 1991

First International Symposium on Combination Therapies, Washington, DC, March 14-15, 1991

Medical Grand Rounds, Yale University School of Medicine, July 11, 1991

PharmaDesign Inc. course on "Cancer-Basic Principles", September 20, 1991

American Society for Histocompatibility and Immunogenetics, Plenary Session speaker, October 13, 1991, Washington, DC.

Dr. Mildred Scheel Foundation, "Metastasis: Basic Research and Clinical Applications," Bonn, Germany, October 28-31, 1991

Radiation Biology Seminar, Thomas Jefferson University, "Immunotherapy of Melanoma with Haptenized Vaccine," January 17, 1992.

Sloan-Kettering Memorial Cancer Center, Melanoma Conference, January 21, 1992.

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Specific Immunotherapy of Cancer with Vaccines, New York Academy of Sciences, Washington, DC, January 21-24, 1993

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American Association for Cancer Research Symposium: "New Approaches to Cancer Immunotherapy", April 11, 1994

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Monoclonal antibodies and tumor vaccines in oncology, Stockholm, Sweden, November 29, 1994

Institut Gustave-Roussy, Villejuif, France - seminar - December 1, 1994

Istituto Tumori, Milan, Italy - seminar - December 2, 1994

Morristown Memorial Hospital, December 19, 1994

Biological Response Modifiers Program, National Cancer Institute, Frederick, MD, January 20, 1995

American Association for Cancer Research, Chairman, Symposium on Tumor Vaccines, March 20, 1995

Interleukin Twelve: Clinical Progress and Future Directions, sponsored by Genetics Institute, Dallas, TX, June 3-4, 1995

October 11, 1995 - Grand Rounds, University of Pennsylvania Cancer Center.

January 18, 1996 - Surgical Grand Rounds, Fitzgerald Mercy Hospital, Darby, PA

Sacred Heart Medical Center, Spokane, Washington - "Current Topics in Malignant Melanoma", March 8, 1996

Surgical Grand Rounds, Hospital of the University of Pennsylvania, March 16, 1996.

Fox Chase Cancer Center seminar, June 19, 1996.

Frontiers in Science Mini Symposium: The Metastatic Cascade, National Institute of Environmental and Health Sciences, Research Triangle Park, NC, June 18, 1996.

University of Illinois Cancer Center Seminar, Chicago, December 13, 1996

Allegheny University, Hahnemann Division - Tumor Conference, February 14, 1997

Fourth International Congress on Biologic Response Modifiers, San Antonio, TX, March 15, 1997

Medical Center of Delaware medicine conference, Wilmington, DE, March 25, 1997

Abington Memorial Hospital, Abington, PA, April 23, 1997

Grand Rounds, University of Pennsylvania Cancer Center, June 18, 1997

Developmental Therapeutics Committee, Meeting of Gynecologic Oncology Group (GOG), July 25, 1997

Cleveland Clinic Foundation Cancer Center, "New Horizons in Diagnosis and Treatment of Cutaneous Malignancies," November 15, 1997.

Department of Radiology Research Conference, Thomas Jefferson University Hospital, January 20, 1998.

National Cancer Center, Tokyo, Japan - February 19, 1998

Tokyo University, Tokyo, Japan - February 20, 1998

Cancer Research Institute, New York, NY, February 23, 1998

Rush Cancer Institute, Rush-Presbyterian-St. Lukes Medical Center, February 24, 1998.

International Quality and Productivity Center Conference on Cancer Vaccines, Bethesda, MD, April 28, 1998

Medical Grand Rounds, Mayo Clinic, Scottsdale, Arizona – October 23, 1998

Grand Rounds, Maricopa Medical Center, Phoenix, Arizona – October 23, 1998

Third International Conference on the Adjuvant Therapy of Malignant Melanoma, London, UK – March 20, 1999

"Ask the Experts" session, American Association for Cancer Research, April, 1999.

Department of Otolaryngology Conference, Hospital of the University of Pennsylvania, July 8, 1999

Hadassah University Hospital, Jerusalem, Israel – Keynote Lecturer at Symposium on "Cancer Vaccines and Immunotherapy", October 26, 1999

Ellis Fischel Cancer Center, "Key Challenges in Clinical Oncology," talks on vaccines for melanoma and ovarian cancer, April 14-16, 2000

Medical College of Ohio, Distinguished Lecture Series, May 1, 2000.

Kimmel Cancer Center, *Comprehensive ASCO Review*, June 1, 2000

Millennial Third World Conference on Vaccines and Immunization - Liege, Belgium, September 1, 2000

Innovative Therapy for Cancer Patients for the New Millennium, New York Medical College, September 12, 2000

Chemotherapy Foundation Symposium XVIII – Innovative Cancer Therapy for Tomorrow. New York, November 11, 2000

College of Physicians and Surgeons of Columbia University, December 4, 2000.

Surgical Grand Rounds, Royal Adelaide Hospital, Adelaide, Australia, April 9, 2001.

Univ. of Connecticutt Health Center, Center for Immunotherapy, April 19, 2001

Japanese Skin Cancer Society, Tokyo, Japan, May 11, 2001.

EDUCATIONAL ACTIVITIES

3/13/89 - Introduction to Clinical Medicine - small group leader

4/5/89 - Judge for Student Research Day, sponsored by Sigma Xi, Thomas Jefferson University.

9/21/89 - "Tumor Immunology, " lecture for Introduction to Immunology, course # MI521, attendance about 50

3/28/90 - Co-Chairman of Student Research Day, sponsored by Sigma Xi, Thomas Jefferson University

6/7/90 - Basic Science Lecture Series, Department of Surgery, Thomas Jefferson University

9/11/90 - Lecture on Tumor Immunology to Clinical Immunology Group

12/4/90 - Medical House Staff Conference - "Lung Cancer"

1/31/91 - Small Group Discussion Leader, Introduction to Clinical Medicine

2/19-3/5/91 - Contemporary Topics in Immunology (MI-626A), 7.5 hours

4/3/91 - Chairman, Student Research Day, co-sponsored by Sigma XI and Division of Graduate Studies

9/19/91 - "Tumor Immunology, " lecture for Introduction to Immunology, course # MI521, attendance about 75

9/30/91 - 11/8/91 - Teaching Attending, Medical House Staff; Internal Medicine Clerkship, Course #350

1/28/92 - Dermatology Residents research conference

2/15/92 - Small Group Discussion Leader, Introduction to Clinical Medicine

9/15/92 - Pulmonary Conference, case discussion, category 1 credit

9/24/92 - "Tumor Immunology, " lecture for Introduction to Immunology, course # MI521

9/28/92 - 11/9/92 - Teaching Attending, Medical House Staff; Internal Medicine Clerkship, Course #350

4/28/93 - Lecture to Clinical Immunology Residents, Department of Pediatrics, Jefferson Park Hospital

5/13/93 - Tumor Immunology Course, Department of Microbiology and Immunology, 3 hours

6/5/93 - Lecture to Class Day, Jefferson Alumni Association, representing class of 1968

6/18/93 - Lecture to Jefferson Network Retreat, Bellevue-Stratford Hotel

8/18/93 - Division of Neoplastic Diseases, Fellows Conference.

9/20/93 - "Tumor Immunology, " lecture for Introduction to Immunology, course # MI521

9/23/93 - Medical Grand Rounds, Thomas Jefferson University, "Melanoma Vaccines: Do They Really Work?"

9/28/93 - Grand View Hospital, Sellersville, PA: CME Program, "Melanoma and Skin Cancer"

4/6/94 - Chestnut Hill Hospital, Clinical Conference, "Melanoma"

7/11/94 - Student Summer Seminar Series (Dr. Chepenik)

7/27/94 - Division of Neoplastic Diseases fellows conference

9/19/94 - "Tumor Immunology, " lecture for Introduction to Immunology, course # MI521

9/23/94 - Grand Rounds, Lankenau Hospital

3/30/95 - Judge, Student Research Day, Thomas Jefferson University

5/23/95 - IM622, Graduate Course in Tumor Immunology, Thomas Jefferson University, 3 hours

6/23/95 - Radiation Biology Seminar, Thomas Jefferson University

7/7/95 - Leukemia Conference, Thomas Jefferson University

7/17/95 - Seminar for summer research students (Dr. Coss)

8/2/95 - Seminar for Division of Neoplastic Diseases Fellows

9/14/95 - "Tumor Immunology, " lecture for Introduction to Immunology, course # MI521

5/30/96. - Medical Grand Rounds, Thomas Jefferson University

7/22/96 - lecture to Thomas Jefferson University summer research students (medical students and undergraudates), invited by Dr. Ron Coss

9/9/96 - "Cancer Vaccines" - Introduction to Immunology (research luncheon lecture)

10/14/96 - Neoplastic Diseases Fellows conference

5/27/97 - IM622, Graduate Course in Tumor Immunology, Thomas Jefferson University, 3 hours

7/14/97 - Seminar for summer research students (Dr. Coss)

10/24/97 - Cardeza Research Seminar

11/19/97 - Jefferson Health System Cancer Registrars educational meeting

2/2/98 - 2/28/98 - Teaching Attending, Department of Medicine, house staff and third year medical students - approximately 10 hours

Introduction to Clinical Medicine, Oncology Section - Course Coordinator - March, 1998 - prepared new syllabus, gave 5 lectures, 3 small groups (2 hours each)

2/9/98-4/27/98 - Introduction to Clinical Medicine, Physical Diagnosis Module - 10 hours 6/18/98 - Fellows' Conference, Depart of Radiation Therapy, Thomas Jefferson University

7/14/98 - Lecture to summer students (Dr. R. Coss' program)

7/30/98 - Lecture to summer research medical students (Dr. K. Chepenik's program)

8/3/98 - Lecture to Medical Residents' Conference (Biological Therapy of Cancer)

12/98 – Introduction to Clinical Medicine, Physical Diagnosis – December, 3,10, 17 – 6 hours

2/23/99 – Department of Dermatology resident's conference.

2/1/99-2/28/99 – Teaching attending, in-patient, Department of Medicine

April, 1999 – Introduction to Clinical Medicine, coordinator of Oncology Section, 5 hours of lecture, 5 hours of small group sessions

5/11/99 – Hobart Amory Hare Society Lecture

5/13/99- Tumor Immunology IM622 – 3 hour session – Department of Microbiology and Immunology

2/1/00 – 2/28/00 – Teaching Attending, Department of Medicine – third year students and house staff – 12 hours

3/30/00 – Introduction to Clinical Medicine, Oncology Small Group Session, 2 hours

6/15/00 – Grand Rounds, Department of Surgery, Thomas Jefferson University

9/11/00 – Division of Medical Oncology Fellows Conference

10/5/00 – Department of Medicine, Medical Residents' Conference

2/22/01 – Cherry Hill Cancer Center – Dr. Howard Kesselheim and group

3/7/01 – Kimmel Cancer Center Grand Rounds

4/30/01 – Tumor Board, Cooper Health Systems, Camden, NJ (Dr. Rocereto)

Effectiveness of Autologous, Hapten-Modified Melanoma Vaccine Depends on the Timing of an Induction Dose. D. Berd, M.J. Mastrangelo, E. Bloome, T. Sato. Division of Medical Oncology, Thomas Jefferson Univ., Philadelphia, PA

We have treated 214 melanoma patients with an autologous vaccine modified with the hapten, dinitrophenyl (DNP) following resection of bulky regional lymph node metastases. Four dosage-schedules were tested, and all included the following: 1)baseline skin-testing (pre-ST) by intradermal injection of $1x10^6$ irradiated autologous tumor cells (AU TC) into the ventral forearm; 2)administration of low dose (300 mg/m^2) cyclophosphamide (CY) iv; 3)multiple intradermal injections of DNP-vaccine (dose range: 2.5-25.0 $x10^6$ irradiated AU TC) mixed with BCG beginning 3 days after CY. One difference between dosage-schedules was the timing of the pre-ST: a)5-7 days prior to CY in 124 patients, b)same day as CY in 27 patients, c)at both time points in 43 patients. Surprisingly, we found a striking difference in clinical outcome between these groups: The 5-year relapse-free survival was *41%* in the group having pre-ST 5-7 days prior to CY vs. *18%* in the two groups with pre-ST on the same day as CY (p=.01, log rank test). Multivariate analysis (Cox regression) showed that this difference was not attributable to an imbalance of known prognostic variables between the groups, *e.g.*., number of (+) nodes. Since we have previously shown that the development of a (+) delayed-type hypersensitivity (DTH) response to unmodified AU TC following vaccine treatment was significantly associated with longer survival, we analyzed the effect of the interval between pre-ST and CY on the induction of post-vaccine DTH. The proportion of patients who developed (+) DTH (≥5 mm diameter induration) was as follows: pre-ST 5-7 days before CY, 81/115= *70.4%*; pre-ST same day as CY, 10/51=*19.6%* (p<.001, Fisher's exact test). These data indicate that what we have considered merely a baseline skin test may serve as an induction dose of vaccine. The timing of the induction dose relative to administration of CY apparently determines whether the subsequent course of DNP-vaccine results in tumor immunity or unresponsiveness.

*1568

Antitumor effects in patients with melanoma, head and neck and breast cancer in a phase I/II clinical trial of interleukin-12 (IL-12) gene therapy. *Hideaki Tahara, Laurence Zitvogel, Walter J. Storkus, Elaine M. Elder, Donna Kinzler, Theresa L. Whiteside, Paul D. Robbins, and Michael T. Lotze. Departments of Surgery, Molecular Genetics and Biochemistry, University of Pittsburgh Cancer Institute, University of Pittsburgh, Pittsburgh. PA.*

IL-12 is a heterodimeric cytokine which induces high-level production of IFN-gamma, promotes Thl type responses and has potent antiangiogenic effects. Injection of the fibroblasts expressing IL-12 genes into murine tumors can eradicate established tumor and elicit systemic anti-tumor immune reaction specific for the tumor at a distant site (Cancer Research 54:, 1994; J. Immunol. 154: 6466, 1995).We initiated a Phase I/II clinical trial of IL-12 gene therapy using direct injection of tumors with genetically engineered autologous fibroblasts based on these promising results. Fibroblast cultures were successfully established from the patients dermal skin, transduced with a retroviral vector expressing human IL-12 (TFG-hIL-12-Neo) and selected with G418. High expression of heterodimeric IL-12 protein from the transduced fibroblasts was observed after selection (usually over 150-200/10^6 cells/24/hours). This protocol was initiated in July of 1995, and the first eighteen patients have been treated with weekly injections of fibroblasts designed to deliver 10, 30, 100, 300, 100 or 300 ng/24 hours in cohorts of 3 patients. 7 breast cancer, 1 thyroid cancer, two head and neck cancer, one colorectal cancer, and 7 melanoma patients have been treated. No untoward effects have been observed. Three patients with recurrent melanoma, one with head and neck carcinoma, one with thyroid carcinoma have been to observed to have shrinkage of the injected lesions as well as distant lesions by more than 50%.

*1569

MUC-1 keyhole limpet hemocyanin (KLH) conjugate plus QS-21 vaccination of high risk breast cancer patients (BCPts) with no evidence of disease (NED). *T Gilewski, R Adluri, S Zhang, A Houghton, L Norton, P Livingston. Memorial Sloan-Kettering Cancer Center, New York, NY.*

Stage IV NED BCPts or earlier stage BCPts NED except for rising CEA or BR2729 levels are at high risk for overt recurrence and might benefit from immunotherapy. Mucin MUC-1, found on most breast carcinomas, is a potential target. A synthetic 30 amino acid (aa) sequence of MUC-1 has been conjugated with KLH and mixed with the immune adjuvant QS21 to increase immune recognition. Nine pts (ages 43-61 years) have been vaccinated: 8 stage IV NED, I stage II with increased CEA level and NED, all but one stage IV NED pt on hormonal tx. All pts received 5 doses of 100 mcg MUC-1 s.c. given on weeks 1, 2, 3, 7, 19. All pts had transient grade 2 local toxicity at the vaccine site and most had grade 1-2 flu like symptoms. All pts remain NED (median follow up 55 weeks) although one pt had a chest wall recurrence which was excised. For all pts the range of IgM and IgG reciprocal titers against purified MUC-1 by ELISA are:

Week #	0	3	5	13	21
IgM	0-160	10-20,480	1280-20,480	10-20,480	320-20,480
IgG	0-10	0-320	40-20,480	160-2560	640-10,240

Five pts maintain IgG titers (range 320-1280) between 6-12 months following the last vaccine. Analysis of IgG subclasses in 8 pts reveal predominantly IgG1 and IgG3. Immune adherence rosetting against MCF-7 cell lines revealed an increase in IgM titers in 6/7 pts. Inhibition assays demonstrate that all sera react exclusively with the APDTRPA determinant of MUC-1. No evidence for augmentation of T cell immunity was found. This MUC-1 vaccine is immunogenic in breast cancer pts who are NED. (Support from NCI PO1 CA33049)

1570

Delayed-type hypersensitivity (DTH) responses induced by autologous, hapten-modified melanoma vaccine – importance of dosage schedule. *D. Berd, M.J. Mastrangelo, E. Bloome, W. Medley, C. Clarke, and H.C. Maguire, Jr. Thomas Jefferson University, Philadelphia PA.*

We have reported that administration of a vaccine consisting of autologous melanoma cells modified with the hapten, dinitrophenyl (DNP-vaccine), prolonged relapse-free and overall survival in patients with clinical stage 3 melanoma following lymphadenectomy. We have compared four dosage-schedules of DNP-vaccine in post-surgical adjuvant patients to determine which are more effective in inducing DTH to autologous, unmodified melanoma cells (autol-MEL): *A*=q 28 days x8, all vaccines DNP-modified; *B*=weekly x 12, alternating DNP-modified and unmodified vaccine; *C*=weekly x12, all vaccines DNP-modified; and *D*=weekly x6, all vaccines DNP-modified. Patients on all schedules except *D* were sensitized to the hapten prior to vaccine. In all four regimens BCG was mixed with the melanoma cells to provide an immunological adjuvant. Dosage-schedules *A* and *D* induced significantly greater DTH to autol-MEL than the more intensive schedules, *B* and *C* (p=.001, Mann-Whitney U test). The proportion of patients who developed a DTH response to autol-MEL ≥5mm was as follows: *A*=20/44=45%, *B*=3/27=11%, *C*=4/22=18%, *D*=16/27=59% (p< .01, Chi square). In contrast, all four dosage-schedules induced similar DTH responses to PPD. Follow-up to date suggests that the two dosage-schedules (*A* and *D*) that were most effective in inducing DTH to autol-MEL produced longer relapse-free survivals than the two schedules (*B* and *C*) that were the least immunologically effective, even after adjusting for standard prognostic variables. Thus, the dose and schedule of administration of human tumor vaccines may be as critical as their composition in inducing immunological responses that have clinical meaning.

1571

A phase I trial of bispecific antibody (BsAB) MDX447 without and with granulocyte colony-stimulating factor (G-CSF) in patients with adult solid tumors. *R.T. Curnow, D.G. Pfister, Y. Deo, R.J. Motzer, C. Winston, K.L. Keeperman, T. Malone. Memorial Sloan-Kettering Cancer Center, New York, NY; Medarex, Inc., Annandale, NJ.*

MDX447 is a BsAB constructed by cross-linking F(ab') fragments of monoclonal antibody (MoAB) H22 to FcγRI and MoAB H425 to the epidermal growth factor receptor (EGFR). In vitro, MDX447 effects lysis of EGFR overexpressing cel1 lines; FcγRI-positive neutrophils (PMNs) constitute a major effector cell population during G-CSF therapy. We have enrolled 26 patients (pts) (median age 54 [38-78]: median Karnofsky 85 [70-90]: male/female 17/9: prior systemic therapy 25: primary cancer kidney 11, head & neck 8, bladder 2, other 3) in a phase I study evaluating MDX447 -/+ G-CSF. Successive groups of 3-6 pts received MDX447 intravenously (IV) weekly (days 1,8,15,22,29,etc.) alone, or with G-CSF (3 mcg/kg/day) subcutaneously (SQ) (days -3 to 1, 4-8, 11-15, etc.). Dose levels of MDX447 evaluated thus far include 1 and 3.5 mg/m^2: 7 mg/m^2 is in progress. Primary toxicities encountered include fever, chills, blood pressure lability, pain/myalgias, and grade 2 increase in 5'nucleotidase: most toxicities abated within 12 hours. Maximum tolerated and biologic dose have yet to be defined. G-CSF induced upregulation of FcγRI on PMNs; in vivo binding of MDX447 to monocytes occurred in all pts, but to a significant degree to the PMNs of only G-CSF treated pts. Of 22 pts evaluable for response, there have been no major responses; 15 pts had stable disease beyond the first month of therapy. Dose escalation continues to better define the dose, toxicity, and potential therapuetic role of this novel biologic.

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PROGRAM/PROCEEDINGS

AMERICAN SOCIETY OF CLINICAL ONCOLOGY

Thirty-Third Annual Meeting

May 17-20, 1997
Denver, CO

Autologous Hapten-Modified Melanoma Vaccine as Postsurgical Adjuvant Treatment After Resection of Nodal Metastases

By David Berd, Henry C. Maguire, Jr, Lynn M. Schuchter, Ralph Hamilton, Walter W. Hauck, Takami Sato, and Michael J. Mastrangelo

Purpose: To determine whether treatment with an autologous whole-cell vaccine modified with the hapten dinitrophenyl (DNP vaccine) is an effective postsurgical adjuvant treatment for melanoma patients with clinically evident nodal metastases.

Patients and Methods: Eligible patients had regional nodal metastases that were large enough (≥ 3 cm diameter) to prepare vaccine. Following standard lymphadenectomy, patients were treated with DNP vaccine on a monthly or weekly schedule.

Results: Of 62 patients with metastasis in a single lymph node bed (stage III), 36 are alive after a median follow-up time of 55 months (range, 29 to 76); the projected 5-year relapse-free and overall survival rates are 45% and 58%, respectively. Of 15 patients with metastases in two nodal sites, five are alive with a median follow-up time of 73 months. An unexpected finding was the significantly better survival of older patients; the projected 5-year survival of patients greater than 50 versus ≤ 50 years was 71% and 47%, respectively ($P = .011$, log-rank test). The development of a positive delayed-type hypersensitivity (DTH) response to unmodified autologous melanoma cells was associated with significantly longer 5-year survival (71% *v* 49%; $P = .031$). Finally, the median survival time from date of first recurrence was significantly longer for patients whose subcutaneous recurrence exhibited an inflammatory response (> 19.4 *v* 5.9 months; $P < .001$).

Conclusion: Postsurgical adjuvant therapy with autologous DNP-modified vaccine appears to produce survival rates that are markedly higher than have been reported with surgery alone. Moreover, this approach has some intriguing immunobiologic features that might provide insights into the human tumor-host relationship.

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PATIENTS WITH MELANOMA metastatic to regional lymph nodes have a relatively poor prognosis, especially when palpable lymph node masses are present.[1-6] Until recently no adjuvant therapy had shown a significant impact on relapse-free and overall survival. The string of negative results was broken by Kirkwood et al,[6] who reported an Eastern Cooperative Oncology Group (ECOG) study of high-dose interferon alfa-2b. Patients who received a 1-year course of interferon following surgery had significantly longer survival than those treated by surgery alone. This was most striking in patients with clinically evident lymph node metastases.

We have developed a novel approach to the treatment of human cancer: immunization with autologous tumor cells modified by the hapten, dinitrophenyl (DNP). Administration of DNP-modified melanoma vaccine has resulted in tumor regression in several patients with measurable metastases.[7] However, like other immunotherapies, the effectiveness is limited by tumor burden, possibly due to the production of immunosuppressive factors, such as interleukin-10, at the tumor site.[8] Therefore, it seemed reasonable to test the DNP vaccine in a setting in which the tumor burden is much lower. Patients with bulky but resectable regional lymph node metastases constitute an ideal group, since the metastatic masses provide a source of cells for preparing vaccine, but the postsurgical tumor burden is below the level of clinical detection. The preliminary results, which have been reported,[9] suggested an unexpectedly long relapse-free and overall survival in patients treated with DNP vaccine after lymphadenectomy. We now present a complete report of those trials, which confirms the preliminary results and provides some insights into the immunobiology of this treatment.

PATIENTS AND METHODS

Patients

The clinical characteristics of the study subjects are listed in Table 1. Sixty-two patients had American Joint Cancer Committee (AJCC) stage III melanoma (CS2, PS2) with at least one clinically evident lymph node metastasis that was at least 3 cm in diameter; the largest nodal metastases were 8 cm in diameter. Six of these patients had in-transit metastases as well. A group of 15 patients who had palpable masses in 2 lymph node sites was analyzed separately. These patients are considered to constitute a much worse prognostic group and, in fact, are sometimes classified as stage IV.[2] All patients underwent standard lymphadenectomy, including, when necessary, exci-

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0732-183X/97/1506-0033$3.00/0

Table 1. Patient-Related Variables

Variable	One Nodal Site (stage III)	Two Nodal Sites (stage IV)
Total patients	62	15
Sex		
Male	36	6
Female	26	9
Age, years		
Median	49	55
Range	21-83	30-72
Nodal site		
Axillary	38	—
Inguinal	19	—
Neck	5	—
Axillary + supraclavicular	—	4
Inguinal + pelvic	—	6
Bilateral axillary	—	3
Bilateral neck	—	2
No. of positive nodes		
1	31	—
2 or 3	20	3
≥ 4	11	12
In-transit metastases	6	0
Primary site		
Extremity	23	5
Trunk	31	8
Head & neck	4	2
Acrolentiginous	2	—
Unknown	2	—
Thickness of primary (mm)		
≤ 1.5	20	5
1.5-3.0	17	5
> 3.0	16	3
NA or unknown	9	2
Time to nodal metastasis		
< 3 months	10	3
3-12 months	18	6
1-3 years	17	1
> 3 years	15	5
HLA type		
$A2^+,A3^-$	15	ND
$A3^+,A2^-$	13	ND
$A2^+,A3^+$	6	ND
$A2^-,A3^-$	28	ND

Abbreviations: NA, not available; ND, not done.

sion of in-transit metastases. Computed tomography (CT) of the thorax, abdomen, and pelvis was performed in the perioperative period, and only patients without definite evidence of metastases were eligible for DNP vaccine treatment. In all cases, the DNP vaccine program was begun within 30 days of lymphadenectomy and the median time from lymphadenectomy was 21 days.

Vaccine Preparation

Tumor masses were obtained within 4 hours of surgery and were processed as previously described.[10] In brief, cells were extracted by enzymatic dissociation with collagenase and DNAse, aliquoted, frozen in a controlled-rate freezer, and stored in liquid nitrogen in a medium that contained 1% human albumin and 10% dimethylsulfoxide (DMSO) until needed. On the day that a patient was to be treated, the cells were thawed, washed to remove DMSO, and irradiated to .25 Gy. Then, they were washed again and suspended in Hanks balanced salt solution without phenol red. Modification of melanoma cells with DNP was performed by the method of Miller and Claman.[11] This involves a 30-minute incubation of tumor cells with dinitrofluorobenzene (DNFB) under sterile conditions, followed by washing with sterile saline.

Each vaccine consisted of 5 to 25 × 10^6 live tumor cells (by trypan blue exclusion) suspended in 0.2 mL Hanks solution; there were variable numbers of residual lymph node lymphocytes in all specimens. After mixing with bacille Calmette-Guérin (BCG) (see later), the suspension was injected intradermally into three contiguous sites. The actual doses of vaccine administered are listed in Table 2.

Study Design

The studies were approved by the Institutional Review Board of Thomas Jefferson University and informed consent was obtained

Table 2. Treatment-Related Variables

Variable	Stage III Schedule A	Stage III Schedule B	Stage IV Schedules A + B
No. of patients	36	26	15
Mean dose per vaccine × 10^6 cells			
(mm)	13	10	10
Median	5-25	2-25	3-17
Range			
No. of vaccine doses administered (mm)	8	12	8
Median	2-8	6-12	1-12
Range			
Peak DTH to unmodified melanoma cells			
(mm)	5	2	4
Median	0-22*	0-6*	0-11
Range	51	8	21
% ≥ 5 mm	9	0	7
% ≥ 10 mm			
Peak DTH to DNP-modified melanoma cells (mm)	20	20	35
Median	7-55	9-70	7-60
Range	100	100	100
% ≥ 5 mm	85	96	90
% ≥ 10 mm			
Peak DTH to DNP-modified lymphocytes (mm)	16	16	15
Median	5-40	5-35	4-40
Range	100	100	93
% ≥ 5 mm	75	69	64
% ≥ 10 mm			
Peak DTH to PPD (mm)	25	23	21
Median	12-60	10-35	13-30
Range	100	100	100
% ≥ 10 mm	71	81	50
% ≥ 20 mm			

*Difference between stage 3, schedule A and stage 3, schedule B: $P = .003$, Mann-Whitney U test.

Table 3. Schedules of DNP Vaccine Treatment

Schedules A and B	
Day −17	Cyclophosphamide
Day −14	DNFB sensitization
Day −13	DNFB sensitization
Day 0	Cyclophosphamide
Schedule A	
Day 3	DNP vaccine + BCG
Day 28	Cyclophosphamide
Day 31	DNP vaccine + BCG
Day 59	DNP vaccine + BCG
Day 87	DNP vaccine + BCG
Day 115	DNP vaccine + BCG
Day 143	DNP vaccine + BCG
Day 171	DNP vaccine + BCG
Day 199	DNP vaccine + BCG
Schedule B	
Day 3	DNP vaccine + BCG
Day 10	DNP vaccine
Day 17	DNP vaccine
Day 24	Unconjugated vaccine + BCG
Day 31	Unconjugated vaccine
Day 38	Unconjugated vaccine
Day 70	Cyclophosphamide
Day 73	DNP vaccine + BCG
Day 80	DNP vaccine
Day 87	DNP vaccine
Day 94	Unconjugated vaccine + BCG
Day 101	Unconjugated vaccine
Day 108	Unconjugated vaccine

from all patients. Two vaccine schedules were tested (Table 3). Schedule A was used for patients accrued from October 1989 to March 1993; schedule B was used for patients accrued from April 1993 to March 1994. For both schedules, patients were initially sensitized with DNFB by topical application of a 1% solution in acetone–corn oil on 2 consecutive days in the same site on the ventral upper arm; cyclophosphamide 300 mg/m^2 by rapid intravenous (IV) infusion was given 3 days before DNFB application.[12] In schedule A, DNP vaccine mixed with BCG (Tice; Organon Teknika Corp, Durham, NC) was administered every 4 weeks for a total of eight doses; cyclophosphamide 300 mg/m^2 was administered 3 days before the first and second doses. All vaccine injections were given in the same site on a limb (usually the upper dorsal arm) that had not been subjected to a lymph node dissection. In schedule B, vaccine was administered weekly for 6 weeks; after a 4-week reevaluation period, vaccine was again administered weekly for 6 weeks. The first three vaccines of each course were DNP-modified and the last three were unmodified. BCG was admixed only with the first and fourth vaccine of each course. All of the DNP vaccine injections were given into one area, and all of the unmodified vaccine injections were given into a second area. Cyclophosphamide 300 mg/m^2 was administered 3 days before the start of each vaccine course.

For both schedules, the dose of BCG was progressively attenuated to produce a local reaction that consisted of an inflammatory papule without ulceration. The attenuation schedule was as follows: (1) 0.1 mL of a 1:10 dilution (1 to 8 × 10^6 colony-forming units [CFU]), (2) 0.1 mL of a 1:100 dilution (1 to 8 × 10^5 CFU); and (3) 0.1 mL of a 1:1,000 dilution (1 to 8 × 10^4 CFU). Because of the progressive development of cell-mediated immunity, most patients were receiving the lowest BCG dose by the fourth BCG injection.

Following the completion of vaccine treatments, patients were evaluated at the Thomas Jefferson University Hospital every 2 months for 2 years, every 3 months for the third year, and every 6 months thereafter. Laboratory evaluations were as follows: complete blood cell count, liver function tests, and chest x-ray every 2 months; and CT of the chest, abdomen, and pelvis every 6 months. No patients were lost to follow-up evaluation.

Skin Testing

Patients were tested for delayed-type hypersensitivity (DTH) by a standard method that we have previously described.[10] Cryopreserved melanoma cell suspensions and peripheral-blood lymphocytes (PBL) were thawed, washed, and irradiated (.25 Gy). Only mechanically dissociated melanoma cell suspensions were used in view of our previous observation that patients immunized with enzyme-dissociated melanoma cells develop strong DTH to collagenase and DNAse.[13] DNP-modified melanoma cells and PBL were prepared as described earlier. A total of 1 × 10^6 melanoma cells and 3 × 10^6 PBL, each either DNP-modified or unmodified, were suspended in Hanks balanced salt solution without serum, phenol red, or antibiotics and injected intradermally into the ventral forearm. The mean diameter of induration was measured after 48 hours. Patients were also skin-tested with intermediate-strength purified protein derivative (PPD; 5 tuberculin units [TU]). DTH testing was performed before DNFB sensitization and then every 2 months during the time of administration of DNP vaccine. Table 2 summarizes the results of DTH testing. No patients exhibited DTH responses to autologous, unmodified PBL, either before or after treatment, which excludes the possibility of spurious responses to the cryopreservation medium or its components.

Human Leukocyte Antigen Testing

Initially, class I typing was performed by the Tissue Typing Laboratory of Thomas Jefferson University Hospital in 20 patients, using standard serologic methods. After preliminary analysis suggested that patients who expressed human leukocyte antigen (HLA)-A3 had a shorter survival than patients with other phenotypes, all of the stage III patients were tested for expression of A3 and A2 by using monoclonal antibodies (American Type Culture Collection, Rockville, MD: A2, HB82; A3, HB122) and flow cytometry. In all cases, the two methods yielded identical results.

Statistics

Survival was plotted by the Kaplan-Meier method and the difference between survival curves was determined by the log-rank test of Mantel. The effect of prognostic variables on survival was determined by proportional hazards regression (Cox).

RESULTS

Stage III: Relapse-Free and Overall Survival

The relapse-free survival of the 62 patients with clinical stage III melanoma treated postlymphadenectomy with DNP-modified vaccine is shown in Fig 1. The median relapse-free survival duration is between 24 and 37 months and the projected 5-year relapse-free survival rate is 45%. All patients have been monitored for at least 29 months and the median follow-up time is 55 months. Of six patients who had in-transit metastases, five are alive and melanoma-free at 44, 45, 60, 60, and 68 months—four continuously relapse-free and one long-term relapse-

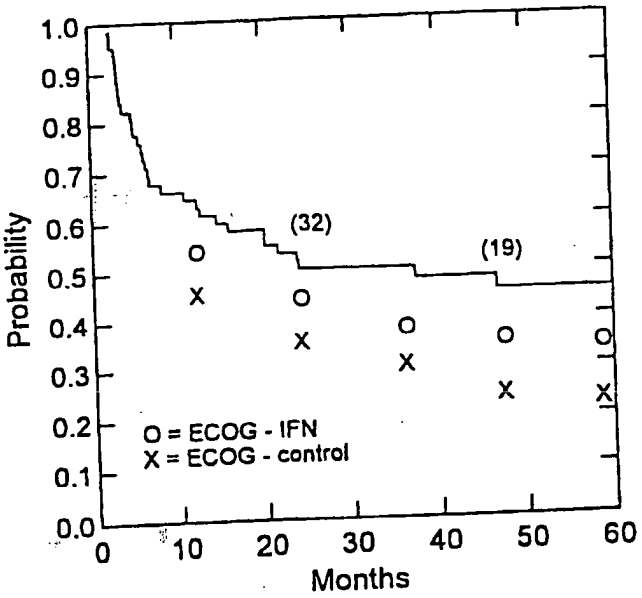

Fig 1. Relapse-free survival of patients with stage III melanoma treated with vaccine. (X, O), relapse-free survival at selected time points of clinical stage III patients treated in the ECOG adjuvant study; (O), surgery alone; (X), surgery + high-dose interferon. (Data from Kirkwood et al.[6])

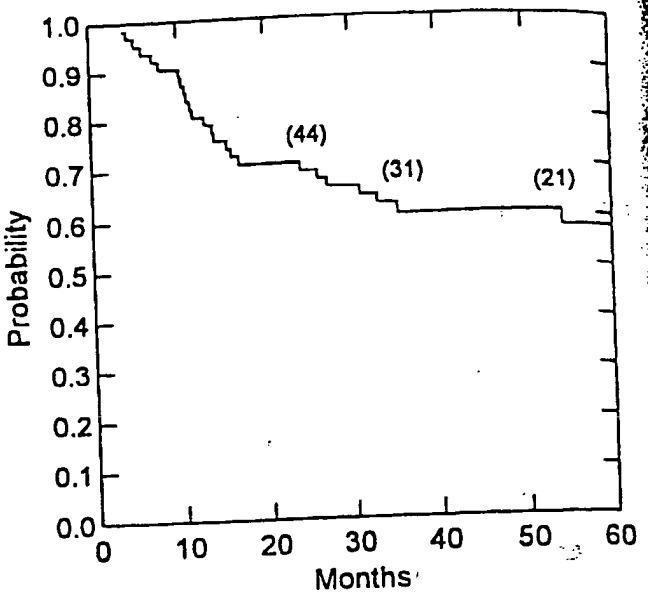

Fig 2. Overall survival of patients with stage III melanoma treated with vaccine.

free after resection of a single recurrence. To provide a comparison with standard therapy, Fig 1 has been annotated to show relapse-free survival at selected points for patients in the ECOG study group who received high-dose interferon alfa-2b and for the untreated (surgery-only) controls.[6] Figure 2 shows the overall survival of these 62 patients. The median overall survival time is more than 62 months and the projected 5-year survival rate is 58%. Since the report by Kirkwood et al[6] did not provide overall survival data for this group of patients, their data could not be shown on this graph.

Univariate Analysis of Prognostic Variables

We performed a univariate analysis of a series of patient-related and treatment-related variables to determine their impact on relapse-free and overall survival of these 62 stage III patients.

Of the patient-related variables examined, age, number of positive nodes, and HLA type had a significant ($P < .05$) impact on survival (Tables 1 and 4). The importance of the extent of nodal involvement is shown in the Kaplan-Meier plot in Fig 3. Patients with a large palpable metastatic mass but no other microscopic nodal involvement had a project 5-year survival rate of 72% versus 34% in patients with four or more positive nodes. The effect of age is shown in Fig 4; older patients (> 50 years) fared significantly better than younger patients. This finding was unexpected, since the ECOG interferon study reported the opposite result,[6] and in other studies, age has been a neutral factor.[2] Even more surprising was the effect of HLA class I phenotype: the 13 patients who were $A3^+A2^-$ had a median survival time of only 11 months, compared with more than 30 months for patients who expressed A2 (with or without A3) or who did not express either A2 or A3. Sex and site or thickness of the primary melanoma were not statistically significant predictors of survival.

It is of particular interest that the interval from treatment of the primary cutaneous melanoma to the time of development of a clinically apparent nodal metastasis was not a significant variable for either relapse-free or overall survival. Patients with a clinically evident nodal metastasis at the time of presentation of the primary melanoma (ie, time to nodal metastasis = 0) have been reported to have a particularly poor prognosis (5-year relapse-free survival rate, < 10%[6]). Of eight such patients in our trial, four are alive at 37, 44, 54, and 67 months, respectively.

Of the treatment-related variables, the development of a positive (≥ 5 mm) DTH response to unmodified autologous melanoma cells was associated with significantly longer 5-year survival (71% *v.* 49%) (Tables 2 and 5; Fig 5). In contrast, the other DTH responses that developed following administration of DNP vaccine (DNP-modified autologous melanoma cells or lymphocytes, PPD) were not predictive, except that the development of a large PPD response was associated with shorter survival, which

Table 4. Univariate Association of Patient-Related Variables With Relapse-Free and Total Survival—Stage III

Factor	No. of Patients	Median Time to Recurrence (months)	P	Median Time to Death (months)	P
Sex			.257		.431
Male	36	22		54	
Female	26	> 16		> 35	
Age, years			.002		.011
≤ 50	35	10		32	
> 50	27	> 47		> 54	
No. of positive nodes			.003		.049
Mass only	31	> 61		> 62	
Mass and 1-2 micrometastes	20	15		35	
Mass and ≥ 3 micrometases	11	5		13	
Primary site			.094		.191
Extremity	23	47		> 54	
Trunk	31	17		36	
Other	8	> 20		> 33	
Thickness of primary (mm)			.771		.569
≤ 2.5	31	37		56	
> 2.5	22	24		> 27	
NA or unknown	9	17		> 33	
Time to nodal metastasis, months			.296		.252
≤ 12	28	37		> 27	
> 12	32	20		54	
HLA type			.003		.001
$A2^+,A3^-$	15	22		> 33	
$A3^+,A2^-$	13	5		11	
$A2^+,A3^+$	6	> 35		> 35	
$A2^-,A3^-$	28	49		> 63	

*Log-rank test.

was of boarderline significance. Schedule of administration (A *v* B) and average vaccine dose were not statistically significant variables.

Multivariate Analysis of Prognostic Variables

A multivariate analysis was performed using a Cox model. Among patient-related variables (Table 6), more extensive nodal involvement was associated with a significantly higher hazards ratio for both relapse-free and overall survival. Age more than 50 years was associated with significantly lower hazards ratios (0.29 and 0.37 for relapse-free and overall survival, respectively). Therefore, the treatment-related variables were analyzed after adjusting for age and number of positive nodes. As shown in Table 7, the failure to develop DTH to unmodified autologous melanoma cells was associated with a hazards ratio of 2.54 for overall survival, but this was of boarderline statistical significance (P = .080). Because of the possibility that patients with more extensive nodal involvement also might be less likely to develop a cell-mediated immune response to melanoma antigens, we modeled DTH to unmodified autologous melanoma cells after adjustment for age only. The hazards ratios for both relapse-free and overall survival increased and were statistically significant (P = .029 and .036, respectively).

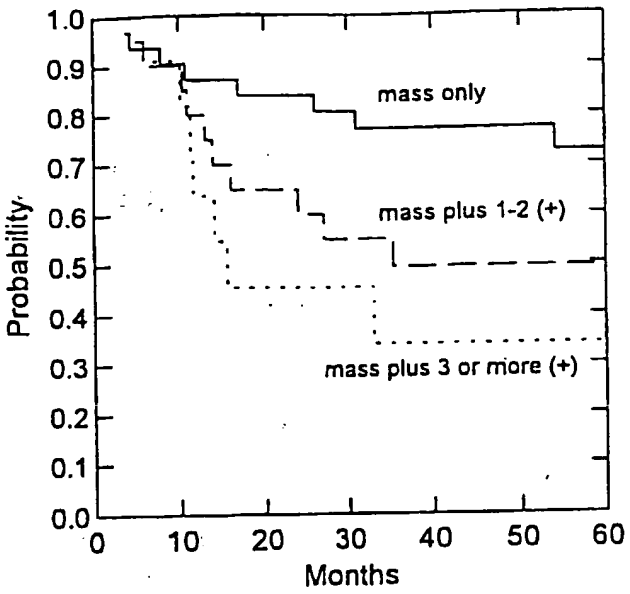

Fig 3. Overall survival of patients with stage III melanoma treated with vaccine stratified by degree of lymph node involvement. P = .049, log-rank test, 2-tailed.

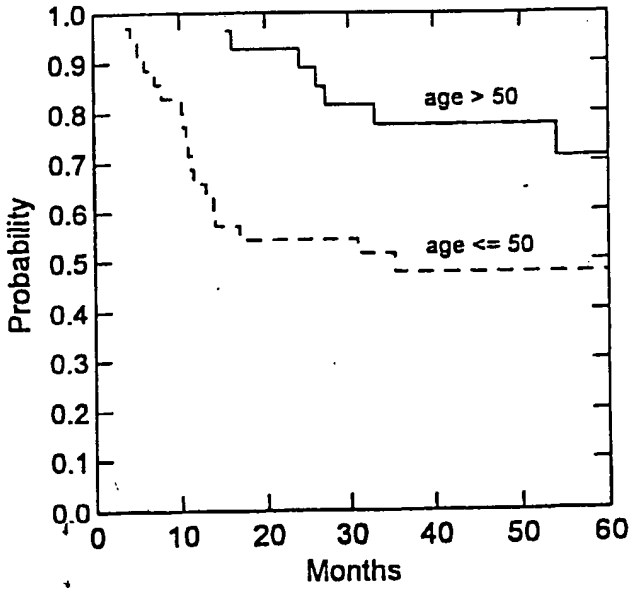

Fig 4. Overall survival of patients with stage III melanoma treated with vaccine stratified by age. P = .011, log-rank test, 2-tailed.

Table 5. Univariate Association of Treatment-Related Variables With Relapse-Free and Total Survival—Stage III

Factor	No. of Patients	Median Time to Recurrence (months)	P*	Median Time to Death (months)	P
Schedule			.265		.512
A	36	46		> 63	
B	26	13		> 27	
Mean vaccine dose			.922		.850
≤ 10×10^6 cells	29	23		54	
> 10×10^6 cells	33	25		> 63	
DTH to unmodified melanoma cells (mm)			.028		.031
≥ 5	19	> 46		> 54	
< 5	38	13		33	
DTH to DNP-modified melanoma cells (mm)			.502		.400
≥ 20	20	12		> 16	
< 20	16	6		24	
DTH to DNP-modified lymphocytes (mm)			.298		.331
≥ 15	31	46		> 62	
< 15	31	16		> 27	
DTH to PPD (mm)			.630		.100
≥ 25	28	24		42	
< 25	28	37		> 35	

*Log-rank test.

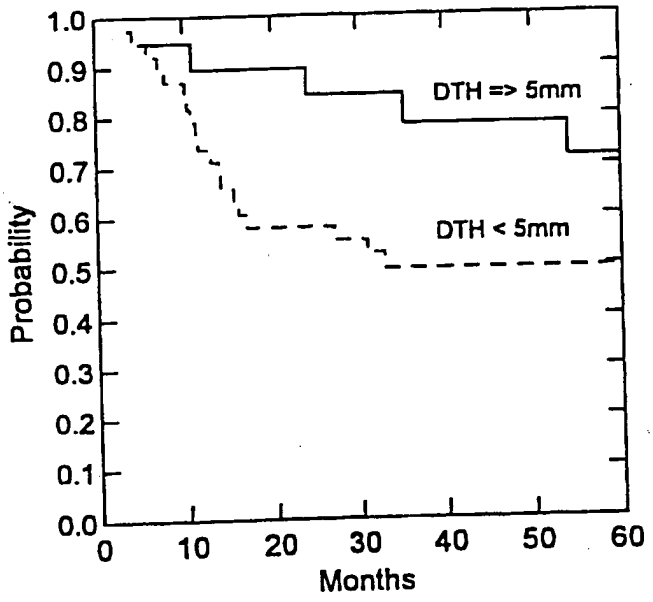

Fig 5. Overall survival of patients with stage III melanoma treated with vaccine stratified by peak DTH to unmodified autologous melanoma cells. $P = .031$, log-rank test, 2-tailed.

The trend toward lower overall survival in patients with large DTH responses to PPD, noted in the univariate analysis, was also evident in the Cox model. This effect disappeared when that parameter was modeled after adjustment for age only, which suggests that it was a reflection of the higher hazards ratios of younger subjects.

It was not possible to generate a Cox model that included HLA type. However, log-rank tests of HLA type stratified by age and number of positive nodes confirmed the results of the univariate analysis of this parameter: HLA type remained a significant predictor of relapse-free and overall survival ($P = .016$ and .014, respectively).

Patients With Metastases to Two Lymph Node Sites

We treated 15 patients with autologous DNP vaccine after resection of bulky metastases to two nodal sites, generally classified as stage IV.[2] Five of 15 are alive and melanoma-free at 32, 39, 73, 76, and 81 months—three continuously relapse-free and two long-term relapse-free after resection of a single recurrence. The sites of nodal metastases in these five patients were as follows: inguinal plus pelvic, n = 3; bilateral axillary, n = 1; and axillary plus neck, n = 1.

Sites and Treatment of Relapses

A total of 46 patients developed recurrence of melanoma following DNP vaccine treatment. The anatomic sites of first recurrence were as follows: soft tissue, n = 26; lung, n = 5; liver, n = 5; bone, n = 4; brain, n = 5, and intraabdominal, n = 1. This distribution of sites of recurrence is similar to that reported in a large retrospective study.[14] It is noteworthy that brain metastases were uncommon, since the brain is considered by some investigators to be an immunologically privileged site that would not be protected by the development of active tumor immunity.[15]

The management of patients following relapse was not specified in the vaccine protocol; instead, patients received what was considered to be standard of care by their physicians. Initial postrelapse treatments were as follows: chemotherapy, 24 patients; surgery (usually complete excision of one or two soft tissue metastases), 18 patients, six of whom subsequently received chemotherapy; and no treatment (because of patient refusal or rapidly progressive metastases), four patients. Of 30 patients who received combination chemotherapy,[16] there were eight partial responses with a median duration of 3 months and no complete responses. Chemotherapy did not appear to have an impact on postrelapse survival. Of 18 patients who underwent surgery, eight were treated with a second course of DNP vaccine, made by excising

Table 6. Multivariate Analysis (Cox model)—Stage III—Known Prognostic Factors

Factor	Relapse-Free Survival			Overall Survival		
	Hazards Ratio*	95% CI	P	Hazards Ratio*	95% CI	P
Sex, female	0.90	0.37-2.21	.822	0.84	0.32-2.25	.733
Age > 50 years	0.29	0.11-0.75	.008	0.37	0.13-1.04	.053
No. of positive nodes (v mass only)			.001			.030
Mass and 1-2 micrometases	2.43	1.06-5.58		2.48	0.99-6.23	
Mass and ≥ 3 micrometases	6.54	2.25-19.02		3.80	1.22-11.84	
Primary site (v extremity)			.565			.174
Trunk	1.07	0.45-2.53		†		
Other	0.36	0.04-3.12				
Thickness of primary (v ≤ 2.5 mm)			.876			.592
> 2.5	0.90	0.39-2.10		0.59	0.22-1.62	
Unknown	1.21	0.49-2.75		0.77	0.20-3.07	
Time to nodal metastasis > 12 months	1.16	0.49-2.75	.730	1.23	0.50-3.01	.647

Abbreviation: CI, confidence interval.

*Hazards ratios with $P < .100$ are highlighted in boldface.

†Results are for Cox analyses, including all variables listed. The exception is that Cox regression could not fit primary site for analysis of overall survival. Therefore, the P value is adjusted only for age and no. of positive nodes. The overall survival results for the other factors are not adjusted for primary site.

and processing the recurrent tumor tissue. Of this group, six have died and two are surviving relapse-free at 25 and 26 months postrecurrence, respectively.

Histology of Recurrent Tumors

Fourteen patients had skin (subcutaneous or dermal) metastases as the initial site of recurrence. Seven of these tumors exhibited inflammatory responses that were histologically similar to those that we have reported in patients who received DNP vaccine for treatment of measurable metastases.[7,17] A representative histology is shown in Fig 6. We calculated the time from first recurrence of melanoma to death and determined whether tumor inflammation was a significant prognostic variable. The median survival times were as follows: inflammation in recurrent tumors, greater than 19.4 months; and no inflammation, 5.9 months ($P < .001$, log-rank test) (Fig 7). A multivariate analysis of prognostic variables for postrelapse survival for this group showed that tumor inflammation was the only statistically significant predictor of longer survival. For example, whether subcutaneous metastases were completely resected was not a significant factor in univariate or multivariate analyses.

Toxicity

Systemic toxicity was almost entirely attributable to cyclophosphamide, and, as expected with the low dose, it was mild. Approximately one third of patients reported short-lived nausea or vomiting, which in most cases was grade 1 or 2. One patient treated on schedule B developed generalized urticaria 15 minutes after injection of her fourth dose of DNP vaccine; this abated spontaneously and was not associated with other symptoms of hypersensitivity. The vaccine was discontinued and this patient has remained tumor-free for 5 years. Otherwise, the toxicity of the DNP vaccine was limited to local reactions at

Table 7. Multivariate Analysis (Cox model)—Stage III—Treatment-Related Variables

Factor	Relapse-Free Survival			Overall Survival		
	Hazards Ratio*	95% CI	P	Hazards Ratio*	95% CI	P
Adjusted for age and no. of positive nodes						
Schedule B	1.67	0.80-3.47	.167	1.44	0.61-3.39	.398
Mean vaccine dose > 10×10^6	1.26	0.60-2.65	.542	1.12	0.48-2.57	.797
DTH to unmodified melanoma cells < 5 mm	2.03	0.82-5.02	.119	2.54	0.87-7.42	.080
DTH to DNP-modified melanoma cells < 20 mm	0.74	0.31-1.76	.494	1.11	0.39-3.14	.848
DTH to DNP-modified lymphocytes < 15 mm	1.19	0.59-2.39	.626	1.01	0.44-2.30	.988
DTH to PPD < 25 mm	0.64	0.30-1.36	.245	0.40	0.16-1.01	.046
Adjusted for age only						
DTH to unmodified melanoma cells < 5 mm	2.49	1.07-5.82	.029	2.81	1.03-7.65	.036
DTH to PPD < 25 mm	0.94	0.46-1.92	.872	0.65	0.26-1.62	.352

*Hazards ratios with $P < .100$ are highlighted in boldface.

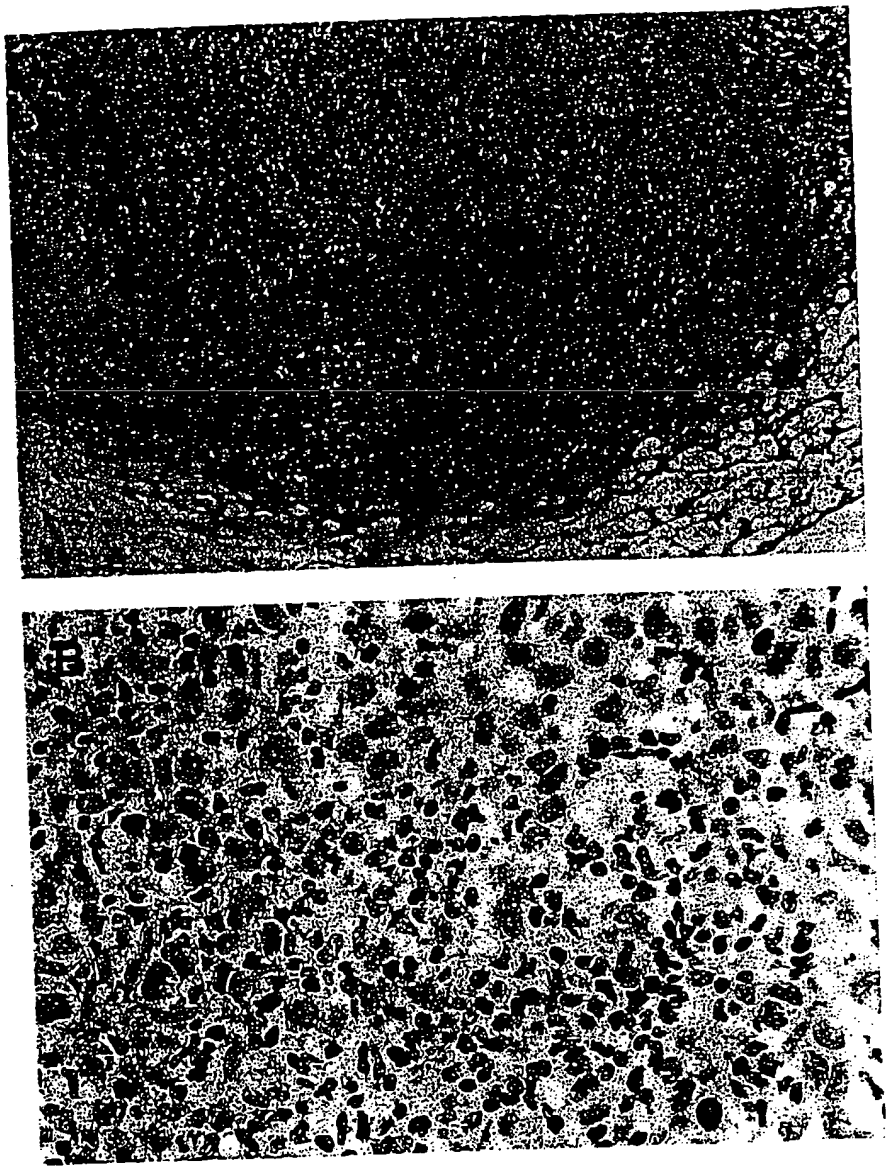

Fig 6. Histology of a subcutaneous metastasis as the first site of melanoma recurrence. Original magnifications: (A) 100×; (B) 400×. Note the dense infiltration of lymphocytes into the tumor tissue. Clinically, this lesion was a 5-mm diameter subcutaneous mass on the mid abdomen.

the vaccine sites. All patients developed pruritic papules that progressed to pustules, sometimes with small ulcerations; the intensity of the reactions was ameliorated by reducing the dose of BCG. No patients noted fever or chills following vaccine administration and no patient experienced a decrease in performance status. Finally, no systemic autoimmune phenomena (eg, arthritis or cutaneous vasculitis) were observed. Vitiligo was never seen.

DISCUSSION

We have developed a novel approach to the treatment of human cancer: immunization with autologous tumor cells modified by the hapten, DNP. The regimen incorporates the administration of low-dose cyclophosphamide before immunization because of its ability to augment cell-mediated immune responses.[10,18] In an initial report, we demonstrated that patients with metastatic melanoma administered a DNP-modified vaccine developed inflammatory responses in metastatic tumor masses.[7] Immunohistochemistry and flow cytometric analysis of biopsy specimens showed infiltration with lymphocytes, the majority of which were $CD8^+$, HLA-DR^+ T cells.[17,19] Polymerase chain reaction (PCR)-based analysis of these tissues indicates that the T cells produce interferon gamma.[20] Recently we, in collaboration with the group

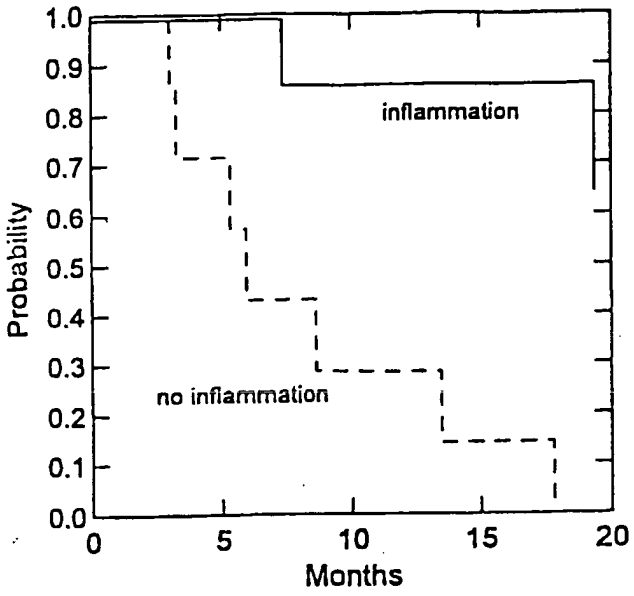

Fig 7. Effect of tumor inflammation on survival from first relapse. Upper curve (——), survival of 7 patients with first recurrence showed an inflammatory response. Lower curve (– – –), survival of 7 patients with first recurrence showed no evidence of an inflammatory response. $P < .001$, log-rank test, 2-tailed.

at the Istituto Nazionale in Milan, have reported that the infiltrating T cells represent expansion of a restricted set of T-cell receptor V-beta gene families, and that in two of two patients studied, the T-cell expansion was clonal.[21] These clones were not found in noninfiltrated metastases excised before administration of DNP vaccine. These observations suggest that immunization with DNP-modified autologous melanoma induces a T-cell response at the tumor site that is driven by yet-to-be-identified melanoma antigens.

The rationale for the use of a hapten-modified human tumor vaccine is well established. It is known that immunization of mice with TNP-modified lymphocytes results in the development of splenic T cells that exhibit secondary proliferative and cytotoxic responses to trinitrophenyl (TNP)-modified cells in vitro.[22] These responses are sometimes associated with low-grade, but reproducible, cross-reactivity with unmodified autologous targets/stimulators. This phenomenon has acquired clinical relevance through the work of Neurath et al,[23] who induced autoimmune colitis in mice by a single application of a hapten to the rectal mucosa. Moreover, it is the basis of drug-induced autoimmune disease: drugs act as haptens, which combine with normal tissue protein-forming immunogenic complexes that are recognized by T cells.[24] Subsequently, autoimmune disease, eg, systemic lupus erythematosus, can develop and continue even after withdrawal of the offending drug.[25,26]

The existence of T cells generated by hapten-modified cells that are reactive to both modified and unmodified cells has recently been directly demonstrated. Ortmann et al[27] have shown that class I major histocompatability complex (MHC)-restricted T-cell clones generated from mice immunized with TNP-modified syngeneic lymphocytes respond to MHC-associated, TNP-modified self-peptides. Furthermore, some TNP-reactive clones respond to certain MHC-binding, unmodified peptides as well.[28] A similar observation has been made with murine T-cell hybridomas responsive to hen egg lysozyme (HEL) modified with the hapten phosphorylcholine (PC).[29] The immunochemical basis of this phenomenon remains speculative, but several hypotheses are being tested. For example, Martin et al[28] have explained their results by hypothesizing the existence of autoreactive T cells that escape thymic selection because of low affinity for self-peptides. Hapten modification of such peptides may convert subdominant peptide epitopes into dominant determinants and thereby activate those T cells.

The current results suggest that administration of autologous DNP vaccine may be an effective postsurgical adjuvant treatment in patients with bulky, regional lymph node metastases. The 5-year relapse-free survival (45%) and overall survival (58%) rates in our series appear to be considerably higher than survival rates achieved with lymphadenectomy alone. Slingluff et al,[4] in a retrospective study of 4,682 patients, found that the "incidence of distant metastases closely mirrored the incidence of regional metastases." This generalization seems to be supported by data from a number of other published reports. For example, Balch et al[1] reported a 5-year survival rate of 24% for patients with clinically detectable nodal metastases. Karakousis et al[3] observed that of 111 patients with palpable positive nodes, only 18% survived 5 years. In the Sloan-Kettering series of 1,019 patients reported by Coit et al,[2] the 5-year survival rate of patients with palpable nodes more than 3 cm in diameter in one nodal site was 22%; when two nodal sites had palpable metastases, the 5-year survival rate decreased to 8%. Finally, in a study reported by Retsas et al,[5] the 5-year survival rate of 169 patients with clinically detectable nodal metastases treated with surgery alone was 28% and the relapse-free survival rate was approximately 10%.

Similar survival statistics have been reported in the ECOG study of interferon alfa-2b as postsurgical adjuvant therapy of melanoma.[6] This landmark study showed that administration of high-dose interferon significantly increased relapse-free and overall survival. The 5-year re-

lapse-free survival rate of surgical controls with clinically evident nodal metastases was approximately 23%, which was increased to approximately 33% with interferon treatment.[6] In our study, administration of DNP vaccine resulted in a relapse-free survival that is twice that of the ECOG control group and somewhat higher than that of the high-dose interferon group.

Although this comparison is weakened by the fact that it is historical rather than prospective, it is strengthened by the inclusion in our trial of patients who would be expected to have a particularly poor prognosis. Eight patients presented with palpable lymph node metastases at the same time that their primary melanoma was diagnosed, an occurrence that has been associated with a 5-year relapse-free survival rate of less than 10%.[6] Six patients had in-transit metastases, as well as clinically evident lymph node metastases. Finally, we did not exclude patients with extranodal extension of melanoma, because tumor processing precluded microscopic evaluation of this feature. In fact, some of the tumors that we processed were quite large (> 5 cm) and may have consisted of smaller, matted nodes—evidence of invasion of the lymph node capsule.

Analysis of the effect of patient-related and treatment-related variables on survival after administration of DNP vaccine disclosed several associations that may be important in understanding the immunology of this therapeutic approach. One striking finding was the superior outcome of older patients. In both univariate and multivariate analyses, patients greater than 50 years of age had significantly longer relapse-free and overall survival times. This is opposite to the age effect noted in the ECOG interferon study, in which older patients had a significantly worse outcome. The reason for our result is, of course, speculative. A provocative but testable hypothesis is that the increased tendency toward autoimmunity associated with aging makes it easier to break tolerance to tumor antigens by active immunization.[30] A peculiar finding was the poor outcome in patients with the HLA phenotype, $A3^+,A2^-$. Since we did not perform complete HLA analysis in our patients, this observation could be dismissed as artifactual. However, before doing so, one should consider that two varieties of putative autoimmune disease occur at a much lower frequency in people who express HLA-A3.[31,32] Thus it is possible that this phenotype is associated with diminished immune responsiveness in certain situations.

Finally, we found that patients who developed DTH to autologous, unmodified melanoma cells had significantly longer survival following treatment with DNP vaccine. This observation strengthens our claim that DNP vaccine works by inducing an antimelanoma immune response. Also, it provides an important immunologic parameter by which the effectiveness of new vaccine approaches can be measured. It is important to note that the magnitude of DTH responses to DNP-modified melanoma cells was uniformly high and was not predictive of clinical outcome. We believe that the development of cell-mediated immunity to hapten-modified cells is necessary, but not sufficient for the generation of an antitumor response.[28]

Little is known about the importance of dosage schedule for the effectiveness of human tumor vaccines. Our original dosage schedule (schedule A) was based on work previously performed with a less successful, nonhaptenized autologous melanoma vaccine.[10] After analyzing the immunologic results of schedule A, we made a strategic decision to test a second dosage schedule (B) that was more intensive and might be expected to induce stronger DTH responses more rapidly. Moreover, in a pilot study of schedule B in melanoma patients with measurable metastases, we observed a striking case of 95% regression of multiple lung metastases (unpublished observation). The results of the current study do not support our hypothesis, because (1) the clinical effects of schedules A and B (ie, relapse-free and overall survival) were not significantly different; and (2) DTH to unmodified autologous melanoma cells was actually greater with schedule A. Thus, at this writing, we cannot recommend one schedule over the other, and additional studies of the immunologic and clinical effects of DNP vaccine dosage and frequency of administration are in progress.

The finding of tumor inflammatory responses in the initial sites of relapse in seven of 14 patients in whom this could be evaluated (ie, skin metastases) was unexpected. To our knowledge, this phenomenon has not been previously reported in either surgical series or in patients receiving adjuvant therapies. The histology of these responses closely resembles the tumor inflammatory responses induced by DNP vaccine in patients with clinically evident metastases,[7,17] and suggests an incipient immune reaction against the recurrent tumor. The observation that inflammation of recurrent tumors was associated with prolonged survival indicates that the response was clinically meaningful.

This is the largest published trial of an autologous tumor vaccine as a postsurgical adjuvant therapy. Although the results must be interpreted with caution in the absence of a concomitant control group, patients who received DNP vaccine appear to have markedly higher relapse-free and overall survival rates than have been reported with surgery alone. Moreover, the survival percentages are comparable to, and may be higher than, those

of patients treated with high-dose interferon, and were achieved with minimal toxicity. Finally, DNP vaccine has some intriguing immunobiologic features—increased effectiveness in older subjects, possible HLA association, induction of tumor DTH responses, and inflammation in recurrent tumor sites—that have not been reported in other human tumor vaccine trials.[33,34]

There are practical considerations for the large-scale testing and application of an autologous melanoma vaccine. The vaccine requires the availability of at least 5 g of tumor tissue (a 2- to 3-cm diameter mass) and a laboratory that is capable of processing it. We have demonstrated the feasibility of acquiring and processing tumor specimens and preparing and administering haptenized vaccines for the treatment of 50 to 75 patients per year. Although the procedures are relatively simple, upscaling them to provide treatment for hundreds or thousands of patients at multiple sites would be a challenging task that would require the resources of a biotechnology company. However, we believe that, given our promising clinical results, it should now be possible to conduct an adequately powered phase III clinical trial to compare DNP vaccine with an established treatment. Plans for such a trial are in progress.

ACKNOWLEDGMENT

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opment of recombinant, BCG-based immunotherapeutic vaccines that express and secrete prostate specific molecules, such as prostate specific antigen (PSA) and prostate specific membrane antigen (PSMA). We have already engineered BCG to intracellularly express 2/3 of the PSA molecule, and are currently testing its ability to generate anti-tumor immunity in mice. As the prostate is a nonessential organ, the immunization of patients against PSMA or PSM, after prostatectomy, may be an opportune time and effective means to eliminate metastases.

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#2427 Mechanism of chemo-immunosensitization of prostate carcinoma target cells to cytotoxic lymphocytes-mediated apoptosis. Ng, C.P., Frost, P., Garban, H., and Bonavida, B. *Department of Microbiology & Immunology, UCLA School of Medicine, 10833 Le Conte Ave., A2-084 CHS, Los Angeles, CA 90095-1747.*

The development of prostate tumor cells resistance to conventional hormonal and cytotoxic therapies has led to the exploration of the immune system in eradicating resistant tumor cells. The success of immunotherapy is predicated on the absence of cross-resistance between drugs and cytotoxic lymphocytes. However, since drugs and cytotoxic lymphocytes can kill by apoptosis, it is likely that drug-resistant tumor cells develop also immune resistance. Recent studies in our laboratory have reported that Fas expressing prostate tumor cells are resistant to Fas-mediated apoptosis. However, the tumor cells can be sensitized to killing following treatment with subtoxic concentrations of cytotoxic drugs (*Uslu et al., Clin. Cancer Res. 3:963, 1997; Frost et al., Cell. Immunol. 180:70, 1997*). We have began exploring the underlying biochemical and molecular mechanisms by which the drugs sensitize the tumor cells to Fas-mediated killing. For instance, pretreatment of adriamycin (ADR)-resistant DU145 prostate cancer cells with ADR sensitizes the cells to Fas-mediated killing. The drug treatment did not modify the levels of Bcl-2 and c-myc (RT-PCR) and FAP-1 and Fas (RT-PCR and Western). Further, there was no induction of Fas-L mRNA or protein. However, there was upregulation of the apoptotic protein, FADD. We have transfected DU145 with neomycin-CMV-driven FADD constructs (sense and antisense) to directly define the role of FADD in ADR-mediated sensitization. Stable transfectants were obtained. We are currently examining these transfectants for sensitivity to Fas as well as the regulation of other genes in the Fas-mediated apoptotic signal.

#2428 Treatment of feline and canine breast cancer with TALL-104 cells. Visonneau, S., Cesano, A., Jeglum, K.A., and Santoli, D. *The Wistar Institute, 3601 Spruce Street, Philadelphia, PA 19104, Veterinary Oncology Services, West Chester, PA 17382.*

The human MHC non-restricted cytotoxic T cell line TALL-104 exerts potent anti-tumor effects in animal models with implanted and spontaneous tumors. The present study tested the ability of TALL-104 cells to prevent relapses in dogs and cats with malignant mammary tumors. Five canine and eleven feline patients at high risk of relapse after surgical removal of the primary tumor and lymph nodes and adjuvant chemotherapy received irradiated (40 Gy) TALL-104 cells (10^8/kg) systemically for 5 consecutive days, followed by monthly boosts. One dog died 16 months later from causes unrelated to cancer, and the other 15 patients are still metastases-free up to 24 months later, despite their poor prognosis (relapse rate = 100% in 6–12 months). No clinical toxicities developed during cell therapy in any of the animals and a strong correlation was found between the patients clinical and immunological responses. Taken together, these data indicate the strong potential of this unique cell line in adjuvant therapy of breast cancer. A Phase I clinical trial in women with metastatic breast cancer is currently underway to determine the maximal tolerated cell dose.

#2429 Tumor-infiltrating lymphocytes (TIL) in breast cancer. Ogawa, Y., Ikeda, K., Kawasaki, F., Nakata, B., Kato, Y., Hirakawa, K., Yoshikawa, K., and Sowa, M. *1st Dept. of Surgery, Osaka City University Medical School, Osaka 545, Japan*

The significance of host immune responses for cancer cells has been reported. To estimate the role of TIL for cancer progression, we analyzed TIL grades in 274 surgically treated patients with breast cancer. TIL of primary tumors were analyzed on H-E stained specimens by microscopic examination and classified into 3 grades as follow: TIL1; no or weak, TIL2; moderate, TIL3; strong infiltration. Higher grades of TIL were seen in tumors growing within the mammary gland compared to those outside (p=.003). And TIL grades increased according to mitosis (p=.012), nuclear pleomorphism (p=.009), Scarff-Bloom-Richardson (SBR) histologic grade (p=.046), and modified SBR nuclear grade (p=.012). Although TIL had no relation to patients age, menopausal status, tumor size, lymph node metastasis, distant metastasis, lymphatic invasion, estrogen receptor, or progesterone receptor status. No difference of disease-free and overall survival was found between patients with and without TIL on primary tumors. In conclusion, poorly-differentiated breast cancer may lead TIL. However, it may be impossible to reveal the significance of host immune responses during the process of tumor progression by TIL.

#2430 Vaccination of breast cancer patients with autologous tumor-associated antigens results in reduction of serum interleukin-6. Jiang, X.P., Head, J.F., Yang, D.C., and Elliott, R.L. *Mastology Research Institute, Baton Rouge, LA 70806*

Serum cytokine measurements could be useful for monitoring patients undergoing immunotherapy. It has been shown for several types of cancer that patients with elevated serum interleukin-6 (IL-6) have a poorer prognosis. Further, patients with progressive renal cell carcinoma with detectable serum IL-6 had a shorter survival from the beginning of IL-2 treatment than patients without detectable serum IL-6. We have previously shown that vaccination of breast cancer patients with autologous tumor-associated antigens (aTAA) mixed with the adjuvants IL-2 and GM-CSF results in an increase in peripheral blood lymphocyte blastogenic response to aTAA and a decrease in the serum level of breast tumor marker CA 15–3. In the present study, serum IL-6 was found to be 0.74±2.51 pg/ml in 36 women being screened for breast cancer, 38.3±122.7 pg/ml in 65 breast cancer patients before vaccination and 13.1±13.2 pg/ml in 37 patients after vaccination. The serum level of IL-6 in breast cancer patients was found to be significantly higher than in the screening group (p=0.017, Welch's t-test). There was a significant decrease (p=0.026, Fisher's exact test) in the proportion of breast cancer patients with serum IL-6 greater than 30 pg/ml from 23.1% (15 of 65 patients) before vaccination to 5.4% (2 of 37 patients) after vaccination. This data shows that vaccination of breast cancer patients with aTAA causes a significant decrease in serum IL-6 concentration and is the first demonstration of a vaccination procedure causing a reduction in serum IL-6.

#2431 Induction of delayed-type hypersensitivity (DTH) to ovarian cancer cells after treatment with an autologous (AUT), hapten-modified vaccine. Berd, D., Carlson, J., Bloome, E., Medley, W., and Dunton, C. *Thomas Jefferson University, Philadelphia, PA 19107.*

Treatment of melanoma patients with a vaccine consisting of AUT tumor cells (TC) modified with the hapten, dinitrophenyl (DNP), induces DTH to DNP-modified TC (DNP-TC) in >95% of patients and to *unmodified* TC in 40–50%. The latter response was a significant predictor of survival in a post-surgical adjuvant study in stage III melanoma. To determine whether these results could be extended to other tumors, we are conducting a study in patients with *stage III ovarian cancer.* Tumor tissue was obtained during surgical debulking, and TC were dissociated and cryopreserved. After 6 cycles of chemotherapy (taxol + cisplatin or carboplatin), patients received 6 weekly doses of DNP-vaccine preceded by a single dose of cyclophosphamide (300 mg/M^2). DTH was tested before treatment and again 2½ weeks after the last vaccine. Of 6 patients treated so far, all have developed positive DTH (≥ 5mm induration) to DNP-TC (median=16mm, range=7–28mm). Unexpectedly all 6 developed DTH to mechanically-dissociated *unmodified* TC as well (median=8mm, range=5–17mm). Thus, AUT DNP-vaccine induces a T cell response in ovarian cancer that is at least equal to that observed in melanoma. This result provides a rationale for a clinical efficacy trial.

#2432 Induction of cytotoxic T cells (CTL) against HLA-A locus-shared lung adenocarcinoma in patients with non-small cell lung cancer (NSCLC). K.Yasumoto, K., Yoshino, I., Takenoyama, M., Hanagiri, T., Imabayashi, S., Eifuku, R., Ichiyoshi, Y. *University of Occupational and Environmental Health, Kitakyushu, Japan.*

To investigate the existence of tumor rejection antigens restricted by the HLA-A locus in NSCLC, the induction of CTL was attempted using HLA-A locus-shared allogeneic NSCLC cells. CTL were induced by the multiple stimulations of T cells derived from either tumor tissues specimens or the regional lymph nodes of patients with NSCLC by an HLA-A2/A24-positive NSCLC cell line (PC-9), and thereafter the CTL activity was examined by a ^{51}Cr release assay. In cases with HLA-A24/adenocarcinoma, CTL were induced in all 11 patients tested. CTL were induced in 10 out of 14 patients with HLA-A2/adenocarcinoma, in 2 out of 3 with HLA-A2/squamous cell carcinoma, and 6 of 8 with HLA-A24/squamous cell carcinoma. These CTL were observed to kill PC-9 selectively, and then the activity was substantially blocked by anti-MHC Class I antibody but not by anti-MHC Class II antibody. The PC-9 specific CTL produced γ-IFN in response not only to PC-9 but also to autologous tumor cells. Furthermore, adoptive transfer of the anti-PC-9 CTL and injection of interleukin-2 effectively suppressed the growth of PC-9 engrafted in SCID mice. These results indicate that PC-9 expresses the T cell epitope(s) presented on HLA-A24 and HLA-A2 that is shared among NSCLC, and potential development of a specific immunotherapy regimen against this disease.

#2433 The study of Induced Killer Cells by combination CD3mAb,IL-2 with soluble colon cacinous antigen. Dong Jing, Zheng Wei-ling. *Department of Immunology, The First Affiliated Hospital, Kunming Medical College, Kunming, Yunnan, Chian, 650032.*

Interleukin-2 (LI-2)-activated Killer Cells are show proliferation, high dependent-LI-2 and poor specificity. It was reported that CD3 monoclonal antibody (CD3mAb) increased the effectiveness of IL-2 Activated Killer Cells. To acquired highly effective, anti-tumor cytotoxicity cells, we combinated CD3mAb, IL-2 with soluble colon cacinous antigen to induce the mononuclear cells of peripheral blood of normal human, we obtained a relative specific tumor antigen activated killer cells (TAK) have cytotocity. We compared TAk with lymphokin activated killer cells (LAK) in proliferation, cytotoxicity and phenotype.